Detection of a Knockdown Resistance Mutation Associated with Permethrin Resistance in the Body Louse *Pediculus humanus corporis* by Use of Melting Curve Analysis Genotyping

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Louse-borne diseases are prevalent in the homeless, and body louse eradication has thus far been unsuccessful in this population. We aim to develop a rapid and robust genotyping method usable in large field-based clinical studies to monitor permethrin resistance in the human body louse *Pediculus humanus corporis*. We assessed a melting curve analysis genotyping method based on real-time PCR using hybridization probes to detect the M815I-T917I-L920F knockdown resistance (kdr) mutation in the paraorthologous voltage-sensitive sodium channel (VSSC) α subunit gene, which is associated with permethrin resistance. The 908-bp DNA fragment of the VSSC gene, encoding the α subunit of the sodium channel and encompassing the three mutation sites, was PCR sequenced from 65 lice collected from a homeless population. We noted a high prevalence of the 3 indicated mutations in the body lice collected from homeless people (100% for the M815I and L920F mutations and 56.73% for the T917I mutation). These results were confirmed by melting curve analysis genotyping, which had a calculated sensitivity of 100% for the M815I and T917I mutations and of 98% for the L920F mutation. The specificity was 100% for M815I and L920F and 96% for T917I. Melting curve analysis genotyping is a fast, sensitive, and specific tool that is fully compatible with the analysis of a large number of samples in epidemiological surveys, allowing the simultaneous genotyping of 96 samples in just over an hour (75 min). Thus, it is perfectly suited for the epidemiological monitoring of permethrin resistance in human body lice in large-scale clinical studies.

The human body louse *Pediculus humanus corporis* (*P. h. corporis*) is a hematophagous ectoparasite that lives and multiplies in clothing. Body lice are vectors of 3 major infectious diseases: epidemic typhus caused by *Rickettsia prowazekii*, relapsing fever caused by *Borrelia recurrentis*, and trench fever caused by *Bartonella quintana* (24). The poor living conditions and crowded situations in homeless, war refugee, or natural disaster victim populations provide ideal conditions for the spread of lice (23). Body louse infestation has been observed in 22% of the sheltered homeless population in Marseille, France, causing trench fever, epidemic typhus, and relapsing fever (4), and coinfestation with head lice is often noted. Consequently, all measures that can be used to decrease the burden of these ectoparasites in homeless people are warranted to avoid the spread and/or outbreak of these diseases. Clothing change as well as the use of ivermectin to eradicate the body lice in a cohort of homeless individuals in Marseille was unsuccessful (10), suggesting that the complete eradication of this ectoparasite is a true challenge (1, 5). It was therefore essential for us to investigate other strategies to reach this goal.

During the 1980s, a study conducted by the U.S. Army demonstrated that the use of uniforms impregnated with 0.125 mg/cm^2 permethrin effectively eradicated body lice (28).

The resistance to permethrin, known as knockdown resistance (kdr), was first identified in the housefly *Musca domestica* (8) and is due to the presence of mutations in the gene encoding the α subunit of the sodium channel that is responsible for the depolarization of nerve cells (9). To date, permethrin resistance has never been studied in *P. h. corporis*. However, in the head louse *Pediculus humanus capitis* (*P. h. capitis*), resistance to permethrin was first reported in France in 1994 and throughout the world thereafter

(11). This resistance is generated by three point mutations, resulting in the amino acid substitutions M815I, T917I, and L920F in the paraorthologous voltage-sensitive sodium channel (VSSC) α subunit (17). According to various authors, these mutations are often found en bloc as a resistant haplotype (7). In Marseille, we wished to conduct a pilot prospective study to eradicate body louse infestations in the homeless individuals frequenting the shelters by providing them with underwear impregnated with a solution of 0.4% permethrin. For this purpose, it was critical to be able to evaluate permethrin resistance before and during the study using a rapid and specific molecular method.

There are currently several different methods available for detecting the mutations responsible for kdr in head lice, including PCR-restriction fragment length polymorphism (PCR-RFLP) (15) and quantitative sequencing (QS), real-time PCR amplification of a specific allele (rtPASA), and serial invasive signal amplification reaction (SISAR) (6). Nevertheless, these methods are time-consuming and not applicable to the investigation of large quantities of arthropods. We report here a rapid and reliable sin-

gle-step method to allow the monitoring of kdr in human body lice.

MATERIALS AND METHODS

Body louse populations. Body lice were collected from volunteers at 2 homeless shelters in Marseille before the beginning of a permethrin clinical trial. The research complied with all relevant federal guidelines and institutional policies (ID RCB: 2010-A01406-33). Alternatively, homeless individuals were given new clothes, and their louse-infested clothing was removed and brought to the laboratory for louse removal. A colony of in-house human body lice maintained on rabbits and never exposed to permethrin has been used as a wild-type control. Two head lice collected in France and Algeria and stored in our laboratory were also used as possibly resistant specimens.

Genomic DNA extraction. Prior to DNA extraction, the collected lice were immersed in 70% ethanol for 15 min and then rinsed with distilled water. Each louse was cut longitudinally into two parts. DNA extraction was performed using a Qiagen tissue kit (Hilden, United Kingdom), according to the manufacturer's instructions. The extracted DNA was assessed for quantity and quality using a NanoDrop instrument (Thermo Scientific, Wilmington, United Kingdom) before being stored at −20°C.

PCR amplification and sequencing of the 908-bp fragment of the VSSC gene. By analogy with head lice, Lee et al. ([17]) hypothesized that acquired resistance to permethrin in body lice was associated with the same mutations. Consequently, we chose to use the already-reported specific primers 5′HL-QS (5′-ATTTTGCGTTTGGGACTGCTGTT-3′) and 3′HL-QS (5′-CCATCTGGGAAGTTCTTTATCCA-3′) to amplify the 908-bp DNA fragment of the VSSC gene ([16]). The Phusion high-fidelity DNA polymerase (Finnzymes, Thermo Scientific, Vantaa, Finland) was used. The reactions were performed in a final volume of 50 μl with 1 U Phusion polymerase, 10 μl 5× Phusion buffer, 0.5 mM (each) primer, 0.16 mM deoxynucleoside triphosphates (dNTPs), and 30 to 50 ng of DNA. The amplification consisted of 35 cycles (98°C for 5 s, 56°C for 30 s, and 72°C for 15 s), preceded by an initial phase at 98°C for 30 s and followed by a termination phase at 72°C for 5 min. The PCRs were performed using the Mastercycler Thermocycler (Eppendorf, Hamburg, Germany). After amplification, all of the PCR products were analyzed by electrophoresis on 1.5% agarose gels using ethidium bromide staining.

Bidirectional DNA sequencing of the targeted 908-bp PCR products was performed using the 3130XL genetic analyzer (Applied Biosystems, Courtaboeuf, France) with the BigDye Terminator v1.1 cycle (Applied Biosystems). The electropherograms obtained for each sequence were analyzed using ChromasPro software. A multiple alignment was performed by the ClustalW method using the Basic Local Alignment Search Tool (BLASTn for nucleotide comparisons) available at http://blast.ncbi.nlm.nih.gov/.

Cloning of the 908-bp DNA fragments. As a high-quality source of target DNA to optimize the test, we amplified DNA fragments from two body lice that had previously been sequenced and shown to be homozygous for the three mutations and cloned them into the pGEM-T Easy vector systems (Promega, Madison, WI) according to the manufacturer's recommendations. The first louse exhibited a wild-type susceptible genotype (SSS), and the second displayed a resistant genotype (RRR). To obtain a heterozygous genotype (HHH), we mixed the two cloned DNA fragments in equal proportions (1:1). R, S, and H represented homozygous resistant, homozygous wild-type, and heterozygous genotypes, respectively. One clone per louse was chosen to conduct our experiments. The plasmids containing the cloned fragments were extracted using the alkaline lysis method ([3]).

Melting curve analysis genotyping. The melting curve analysis genotyping method is based on real-time PCR with hybridization probes, using fluorescent resonance energy transfer (FRET) technology. Two sets of primers were designed to amplify specific regions: (i) a 144-bp genomic fragment of the first exon to characterize the M815I (ATG>ATT) mutation and (ii) a 203-bp fragment of the third exon to detect the 2 other mutations, T917I (ACA>ATA) and L920F (CTT>TTT). In addition to the two primer pairs, two hybridization probes, an anchor probe and a reporter probe, were designed by Tib Molbiol (DNA Synthesis Service, Berlin, Germany) to detect each of the three targeted mutations (see Table S1 in the supplemental material). These probes are alternatively labeled with fluorescein and LC Red 640 fluorochromes. The probes hybridize head to tail to the region containing the mutation site to genotype, allowing the transfer of the fluorescence energy of fluorescein to that of Light Cycler Red 640 (Fig. 1). The emitted fluorescence is measured continuously during the melting phase, during which the temperature is gradually increased, allowing the determination of the melting temperature (T_m) of the reporter probe. The reactions were performed using a LightCycler 480 (Roche Diagnostics Corp.) in 96-well plates (LightCycler Multiwell Plate 96). In a final volume of 20 μl, 10 μl 2× QuantiTect Probe PCR Master Mix (Qiagen), 0.5 mM (each) primer, 0.2 mM anchor probe, 0.2 mM probe reporter, and between 5 and 20 ng DNA were mixed. Two different programs were used for the detection of three mutations in this study (see Table S2 in the supplemental material).

Data analysis. The sensitivity and specificity of the melting curve analysis genotyping method were tested using a contingency table representing the findings of the method test compared to the gold standard of sequencing. The statistical analyses were performed using SPSS for Windows, version 17.2.

RESULTS

PCR amplification and sequencing of the 908-bp fragment of the VSSC gene. A total of 65 lice, including 52 body lice and 1 head louse from homeless persons, 11 body lice from the laboratory colony, and 1 head louse from a schoolchild in Algeria, were used for this assay. The concentration of genomic DNA extracted from the lice varied from 10 to 50 ng/μl.

The 908-bp DNA fragment of the VSSC gene encoding the α subunit of the sodium channel was successfully amplified in all 65 lice tested. Direct sequencing and multiple alignments of these fragments allowed us to deduce the complete reference sequence. Four introns of various sizes (87, 86, 88, and 85 bp) and three exons (141, 174, and 163 bp) were found (see Fig. S1 in the supplemental material). The genotyping results of the 3 mutation sites in the 65 lice tested are summarized in Table 1, with several different profiles identified. Of the specimens genotyped using the Sanger sequencing method and designated RG (reference genotype), 31% exhibited a resistant haplotype (RRR), 29% showed an RHR haplotype, 22% showed an RSR haplotype, and 18% showed a wild-type haplotype (SSS). An alignment of the 65 obtained sequences indicated that all of the lice tested, which contained at least one of the 3 target mutations, also had a single nucleotide polymorphism in the second intron, at approximately nucleotide (nt) 2484 + 4, A> T (data not shown).

Melting curve analysis genotyping. The real-time PCR technique was first improved by testing the known genotype clones. Each of the three mutations was targeted individually. The results obtained with the melting curve analysis and sequencing were superimposable for the three mutation sites. As expected, the wild-type allele showed a peak with a higher T_m than that of the mutated allele because the detection probe complementary to the mutated allele was detached earlier in the presence of a mismatch during the melting step. In addition, the T_m of the duplex probe/mutated codon is lower than that of the duplex probe/wild-type codon. A double peak is characteristic of heterozygotes (see Fig. S2 in the supplemental material).

Thus, the G>T transversion responsible for the replacement of a methionine with an isoleucine at position 815 and the C>T
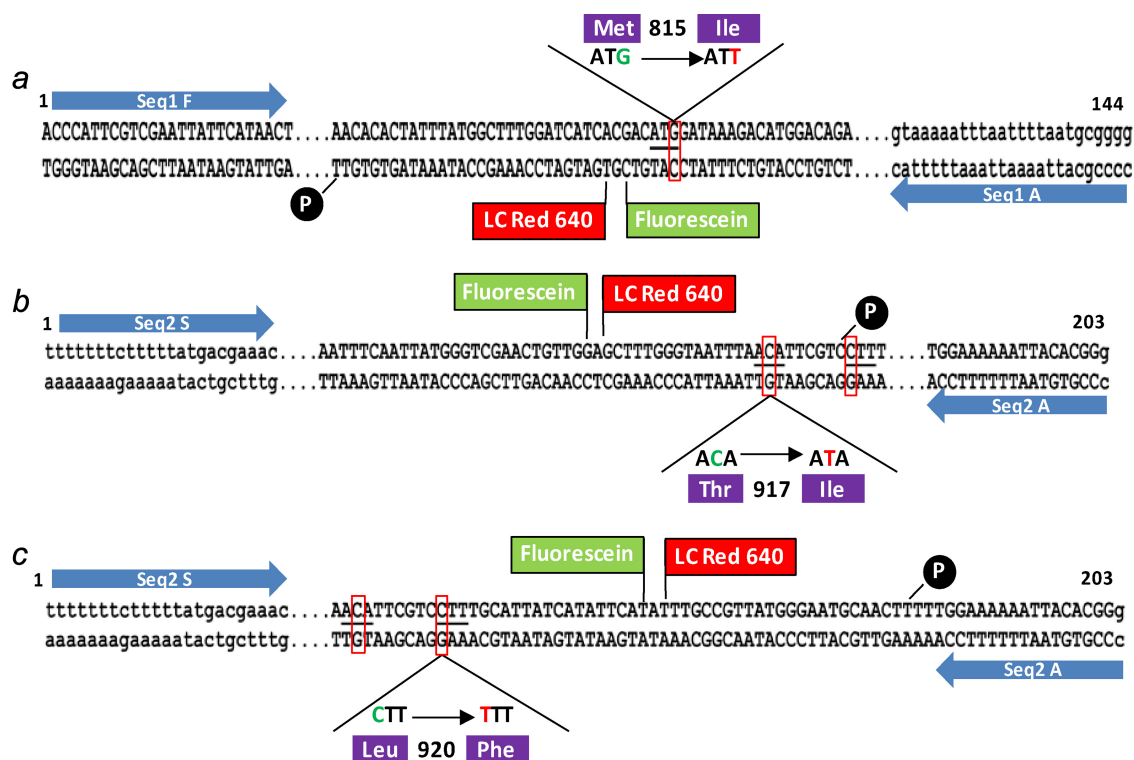
FIG 1 Schematic diagram showing the hybridization of the FRET probes. For the detection of the kdr mutations, M815I (ATG>ATT) in the first exon and T917I (ACA>ATA) and L920F (CTT>TTT) in the third exon, 2 amplicons (a to c) spanning the region containing the polymorphic sites are amplified with 2 sets of primers (primers Seq1 F [forward] and Seq1 A [reverse] [a] and primers Seq2 S [forward] and Seq2 A [reverse] [b and c]).

TABLE 1 Validation of the method of genotyping by comparing the results of melting curve analysis (predicted genotype) and Sanger sequencing (reference genotype)[a]

Louse type (no. of lice tested)	Phenotype	No. of lice in exon with mutation, determined by method					
		First exon, M815I, ATG>ATT		Third exon			
				T917I, ACA>ATA		L920F, CTT>TTT	
		PG	RG	PG	RG	PG	RG
Body lice from homeless (52)	S	0	0	13	13	1[c]	0
	H	0	0	18	19	0	0
	R	52	52	21[b]	20	51	52
Head louse from homeless (1)	S	0	0	0	0	0	0
	H	0	0	1	1	0	0
	R	1	1	0	0	1	1
Human body lice bred on rabbits (11)	S	11	11	11	11	11	11
	H	0	0	0	0	0	0
	R	0	0	0	0	0	0
Head louse from Algeria (1)	S	1	1	1	1	1	1
	H	0	0	0	0	0	0
	R	0	0	0	0	0	0

[a] Abbreviations: PG, predicted genotype obtained by melting curve analysis genotyping method; RG, reference genotype obtained by the Sanger sequencing method; R, resistant homozygote in which both alleles are mutated; S, susceptible homozygote in which neither allele is mutated; H, heterozygote in which only one of the two alleles is mutated.
[b] False-positive result.
[c] False-negative result.

transitions causing the replacements of a threonine with an isoleucine at position 917 and a leucine with an phenylalanine at position 920 each shift the T_m by 7 to 8°C.

To ensure that the results reflected the targeted fragments, we have verified the PCR products by sequencing after genotyping (data not shown).

We then focused on the 65 lice for which the sequence of interest was previously determined using the Sanger sequencing method. The genotypes for each louse obtained by the melting curve analysis genotyping, designated PG (predicted genotype), are reported in Table 1.

Sensitivity and specificity of the real-time PCR with hybridization probe technique. Of the 195 tests performed, only two results were discordant with those obtained by the reference method: a false positive for the T917I mutation and a false negative for the L920F mutation (Table 1). The calculated sensitivity was 100% for the M815I and T917I mutations and 98% for the L920F mutation; the specificity was 100% for M815I and L920F and 96% for T917I.

DISCUSSION

In contrast to head lice, for which the kdr frequency was sometimes as high as 0.90, the permethrin resistance of the body lice was still unknown. Consequently, to promote the eradication of body lice in the homeless population, we have planned a clinical study using permethrin-impregnated underwear. Monitoring the resistance of body lice to permethrin in the homeless population before and during the clinical trial was determined to be mandatory. To achieve this goal, we opted to use a robust and simple

single-step melting curve analysis genotyping method. Since its establishment in 1997 (19), this method has been particularly useful in detecting known mutations causing human diseases such as cancer (27) and other disorders (30). The melting curve analysis genotyping technique is also used in other fields, such as molecular haplotyping (21), bacteriology (22), parasitology (26), and virology (25). Using this method allowed us to determine the genotype of human lice at each mutation site. Furthermore, its ability to detect heterozygotes makes it particularly useful for following the dynamics of the kdr mutation in a targeted population of lice. Despite the two inaccurate results, a false positive and a false negative for the T917I and L920F mutations, respectively, in our total of 195 tests, the melting curve analysis genotyping method proved to have great sensitivity (98% to 100%) and excellent specificity (96% to 100%) in the detection and characterization of allelic resistance to permethrin in human lice. The false-positive result could be due to differences in the strength of probe binding (2) or polymerization errors during the insertion of nucleotides by the *Taq* polymerase during real-time PCR. The resequencing of this sample confirmed its initial genotypic status, which was heterozygous. Conversely, the false-negative result could have been due to a manipulation error during the distribution of the DNA samples or the reagent mixtures into the 96-well plates. Several tools described in the literature are available to monitor both phenotypic and genotypic resistance traits in head lice. In addition to being faster than conventional phenotypic bioassays, which are tedious and not adapted to such large-scale purposes (18), these techniques can simultaneously detect the allele frequencies and point mutations associated with resistance to permethrin without gene sequencing (14).

The eradication of body lice in the homeless is a major challenge. We implemented several different clinical trials during the past 10 years to fight louse infestations without success (4). Although resistance to DDT was reported at the end of the 1940s and in the early 1950s in the United States, southeast Asia, Egypt, and Iran (13, 20), to the best of our knowledge, no data are available for permethrin resistance in *P. h. corporis*. In this work, we noted a high prevalence of the 3 indicated mutations in the body lice collected from homeless people (100% for the M815I and L920F mutations and 56.73% for the T917I mutation). As suggested for head lice, under the selective pressure of permethrin, the mutations M815I and L920F are the first to take place. This event would be followed by the onset of the T917I mutation (12), which is considered the main cause of this resistance, while the M815I and L920F mutations would reduce permethrin sensitivity (29). These findings suggest that the population of body lice infesting homeless people in Marseille has been subjected to selective pressure. Overuse of synthetic pyrethroids to eradicate the various insect pests, notably the increasing infestation with bedbugs in the last 4 decades, has been well documented and probably accounts for the level of resistance reported here.

The presence of a single nucleotide polymorphism (SNP), at approximately nt 2484 + 4, A>T, at the beginning of the second intron of the 908-bp target sequence of the VSSC gene, was an unexpected finding. This polymorphism was found exclusively in those lice that displayed at least one of the 3 tested mutations and could represent an informative SNP for the presence or absence of these mutations; however, further investigations are required to address this possibility.

In conclusion, melting curve analysis genotyping is a fast, sensitive, and specific tool that is fully compatible with the analysis of a large number of samples in epidemiological surveys, allowing the simultaneous genotyping of 96 samples in just over an hour (75 min). Therefore, this method is perfectly suited to the epidemiological monitoring of permethrin resistance in large-scale clinical studies.

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